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Phytochemical investigation and biological activities of extracts obtained from *Montrichardia linifera* (Arruda) Schott leaves collected in different localities from Piauí coast

Investigação fitoquímica e atividades biológicas de extratos obtidos das folhas de *Montrichardia linifera* (Arruda) Schott coletadas em diferentes localidades do litoral piauiense

Investigación fitoquímica y actividades biológicas de extractos obtenidos de hojas de *Montrichardia linifera* (Arruda) Schott recolectadas en diferentes localidades de la costa de Piauí

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Abstract
Montrichardia linifera is an aquatic macrophyte widely used by the population, presenting antibacterial, antioxidant, antiplasmodic, insecticidal and antimalarial activities. This study aimed to evaluate the phytochemical composition and biological activities of extracts obtained from *M. linifera* leaves collected in different locations. Hexanic (HXE), hydroalcoholic (HAE) and methanolic (ME) extracts were prepared from leaves from four collection points in the state of Piauí, Brazil: Porto dos Tatus (PT), Lagoa do Bebedouro (LB), Galego (G) and Morros da Mariana (MM). Qualitative and quantitative phytochemical assays, evaluation of *in vitro* cytotoxicity against three tumor cell lines (HL-60, HCT-116 and B16-F10), evaluation of antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* and analysis of antioxidant potential by DPPH were carried out and ABTS. The extracts showed the presence of alkaloids, organic acids, flavonoids, phenols and tannins, with their presence varying according to the place of collection. The HXE-G sample had the highest percentage of flavonoids. The ME-G and HAE-G samples had the highest levels of phenolic compounds. The HXE-G, HAE-G and ME-G extracts showed a high percentage of inhibition against the three tumor strains tested. HXE-MM had a bacteriostatic effect against both strains of bacteria tested. The samples that showed better antioxidant activity were ME-G and HAE-G. These results demonstrate that the plant has a phytochemical variability, depending on the geographic location and the type of solvent used in the extraction process. Thus, the extracts obtained from *M. linifera* leaves present a variety of secondary metabolites with potential biological activity.
Keywords: Secondary metabolites; Natural products; Aquatic macrophyte; Plant extract.

Resumo
Montrichardia linifera é uma macrófita aquática muito utilizada pela população, apresentando atividades antibacteriana, antioxidante, antiplasmódica, inseticida e antimalárica. O presente trabalho teve como objetivo avaliar a composição fitoquímica e atividades biológicas de extratos obtidos de folhas de *M. linifera* coletadas em diferentes locais. Foram preparados extratos hexânico (HXE), hidroalcoólico (HAE) e metanólico (ME), das folhas de quatro pontos de coleta no estado do Piauí, Brasil: Porto dos Tatus (PT), Lagoa do Bebedouro (LB), Galego (G) e Morros da Mariana (MM). Foram realizados ensaios fitoquímicos qualitativos e quantitativos, avaliação da citotoxicidade *in vitro* contra três linhagens de células tumorais (HL-60, HCT-116 e B16-F10), avaliação da atividade antibacteriana contra *Escherichia coli* e *Staphylococcus aureus* e análise do potencial antioxidante por DPPH e ABTS. Os extratos mostraram a presença de alcalóides, ácidos orgânicos, flavonóides, fenóis e taninos, com sua presença variando de acordo com o local de coleta. A amostra HXE-G apresentou o maior percentual de flavonóides. As amostras ME-G e HAE-G obtiveram os maiores níveis de compostos fenólicos. Os extratos HXE-G, HAE-G e ME-G apresentaram alta porcentagem de inibição contra as três linhagens tumorais testadas. HXE-MM teve um efeito bacteriostático contra ambas as cepas de bactérias testadas. As amostras que apresentaram melhor atividade antioxidante foram ME-G e HAE-G. Esses resultados demonstram que a planta possui uma variabilidade fitoquímica, dependendo da localização geográfica e do tipo de solvente utilizado no processo de extração. Assim, os extratos obtidos das folhas de *M. linifera* apresentam uma variedade de metabólitos secundários com potencial atividade biológica.
Palavras-chave: Metabólitos secundários; Produtos naturais; Macrófita aquática; Extrato vegetal.

Resumen
Montrichardia linifera es una macrófita acuática muy utilizada por la población, que presenta actividad antibacteriana, antioxidante, antiplasmódica, insecticida y antipalúdica. Este estudio tuvo como objetivo evaluar la composición fitoquímica y las actividades biológicas de extractos obtenidos de hojas de *M. linifera* recolectadas en diferentes localizaciones. Se prepararon extractos hexánicos (HXE), hidroalcohólicos (HAE) y metanólicos (ME) a partir de hojas de cuatro puntos de recolección en el estado de Piauí, Brasil: Porto dos Tatus (PT), Lagoa do Bebedouro (LB), Galego (G) y Morros da Mariana (MM). Se realizaron ensayos fitoquímicos cualitativos y cuantitativos, evaluación de la citotoxicidad *in vitro* frente a tres líneas celulares tumorales (HL-60, HCT-116 y B16-F10), evaluación de la actividad antibacteriana frente a *Escherichia coli* y *Staphylococcus aureus* y análisis del potencial antioxidante por DPPH. y ABTS. Los extractos mostraron la presencia de alcaloides, ácidos orgánicos, flavonoides, fenoles y taninos, variando su presencia según el lugar de recolección. La muestra HXE-G tuvo el mayor porcentaje de flavonoides. Las muestras de ME-G y HAE-G tenían los niveles más altos de compuestos fenólicos. Los extractos HXE-G, HAE-G y ME-G mostraron un alto porcentaje de inhibición contra las tres cepas tumorales probadas. HXE-MM tuvo un efecto bacteriostático contra ambas cepas de bacterias probadas. Las muestras que

mostraron mejor actividad antioxidante fueron ME-G y HAE-G. Estos resultados demuestran que la planta tiene una variabilidad fitoquímica, dependiendo de la ubicación geográfica y el tipo de solvente utilizado en el proceso de extracción. Así, los extractos obtenidos de hojas de *M. linifera* presentan una variedad de metabolitos secundarios con potencial actividad biológica.
Palabras clave: Metabolitos secundarios; Productos naturales; Macrófito acuático; Extracto vegetal.

1. Introduction

Natural products have been used for a long time to treat and relieve the symptoms of different diseases once they are sources of bioactive compounds (Mattos et al., 2018; Pio et al., 2019). The ethnobotanical research is the main strategy to develop new phytotherapeutic drugs using natural products, as well as to investigate their uses in the treatment of different diseases (Calixto, 2019; Moura et al., 2019). In this aspect, several studies have proven the biological potential of many medicinal plants, highlighting the necessity to analyze their active components, which may be associated with their biological activity (Reid et al., 2018; Wyk & Wink, 2018).

Brazil has a wide flora and fauna biodiversity, which represents a valuable source of natural products with a great potential for the development of phytotherapy (Cardoso et al., 2018; Honório et al., 2019). The Parnaíba Delta, located between the states of Piauí and Maranhão, corresponds to one of the richest Brazilian environments in terms of biodiversity and is considered a biological sanctuary for innumerable migratory species. Besides, the region also displays a relevant touristic, scientific, and cultural role (Guzzi, 2012; Júnior & Macedo, 2016). Considering the vegetation, hundreds of genera and species, besides dozens of families, have already been identified. Among these, the Araceae family is one of the most representative families identified (Guzzi, 2012).

The species *Montrichardia linifera* (Arruda) Schott, popularly known as Aninga, belongs to the Araceae family and is widely found in the Parnaíba Delta region. This plant is an amphibious aquatic macrophyte, which is found in tropical regions and develops in waterlogged soils (Silva et al., 2013). It is widely used in the therapeutics of the local community as a wound healing, to stop bleeding, and in the treatment of dermatophytosis. Its leaves are considered anti-rheumatic, and its roots, although toxic, are used as an antidiuretic (Macedo et al., 2005; Fenner et al., 2006; Amarante et al., 2009; 2011). Studies have demonstrated that the extracts obtained from the leaves of *M. linifera* presented antibacterial activity against Gram-positive strains (Miranda et al., 2015). The extract obtained from its stem, in turn, showed activity against Artemia salina and antiplasmodial potential (Amarante et al., 2011). Extracts obtained from *M. linifera* collected during the dry season have shown high antioxidant, antibacterial, insecticide, and cytotoxic potentials (Santos et al., 2014). Besides, its phytochemical profile shows different classes of compounds, which have already been reported as anticancer in the literature. These compounds comprise alkaloids (Barrales-Cureño, 2015), flavonoids (Martinez-Perez et al., 2014), phenols (Abdal Dayen et al., 2016; Moore et al., 2016), and triterpenes (Almeida et al., 2009), confirming the biological potential of the species. However, the phytochemical profile of the plants is affected by environmental conditions, such as seasonality and variations in water and soil composition (Ren et al., 2017; Ifie et al., 2018). These variations may interfere with the biological potential of the plants once their phytochemical composition is directly associated with their biological activities (Ren et al., 2017).

In this aspect, this work aimed to investigate the phytochemical composition and the biological potential of different extracts obtained from the leaves of *M. linifera*, collected in different localities of the state of Piauí, Brazil. Such information may clarify the phytochemical profile of the plant used by the local population in different applications, as well as to provide data to future studies.

2. Methodology

This study is an experimental laboratory research, using referenced methodologies, described below.

2.1 Collection and preparation of the plant material

The specimens of *M. linifera* were collected during the rainy season, between March and May 2013, in four locals: Galego (2°46'49''S; 41°51'51''W), Porto dos Tatus (2°50'17''S; 41°49'52''W) and Morros da Mariana (2º51'15,9''S; 41º48'49,1''W), located at Ilha Grande (Piauí, Brazil), and Lagoa do Bebedouro (2°46'49''S; 41°51'51''W), in Parnaíba (Piauí, Brazil). An exsiccate was deposited at Parnaíba Delta Herbarium (HDELTA), from the Universidade Federal do Delta do Parnaíba (UFDPar), under the following protocol number: 3541. The access activity to the Genetic Heritage/CTA was duly registered in SisGen, with the registration number **A3D47B**. The species is represented in Figure 1.

Figure 1. Image of the species *Montrichardia linifera*, collected in Morros da Mariana, Ilha Grande (Piauí, Brazil). The species is an aquatic macrophyte, with several applications in popular medicine.

Source: Authors (2022).

2.2 Extracts preparation

The extraction process was performed according to the method proposed by Marques (2005), with some modifications. The leaves collected were first washed with flowing water, then with distilled water, and submitted to drying in a hot air oven at 50 ºC. After this process, the leaves were crushed with a large-scale grinder. Subsequently, solutions of 1:10 (w/v) were prepared by adding the crushed material to hexane, methanol, or an ethanol-water solution (1:5; v/v). The samples were protected from the light and left under constant stirring, for 24 hours. At the end of the process, the materials were filtered and completely dried in a water bath, at 60 ºC. This procedure was carried out to each element, resulting in 12 samples, which comprised hexanic extracts (HXE), methanolic extracts (ME), and hydroalcoholic extracts (HAE), from the four collection sites: Porto dos Tatus (PT), Lagoa do Bebedouro (LB), Galego (G), and Morros da Mariana (MM).

2.3 Qualitative phytochemical assay

The qualitative phytochemical analysis was performed according to the method suggested by Barbosa et al. (2004), with some adaptations. The assay consisted of a colorimetric method used to detect the presence of secondary metabolites, such as alkaloids, phenols, flavonoids, polysaccharides, saponins, and tannins. The results were determined by the observation of the presence or absence of precipitate and color variations through the addition of specific solvents, such as distilled water and hydrochloric acid (HCl), using two milligrams of the dry extract. For a better solubilization of the hexanic extracts in the assays that requested dilution in water, 1% of hexane (v/v) was added to the samples.

2.4 Phytochemical quantification of flavonoids and total phenolic compounds

2.4.1 Determination of total flavonoids content

Total flavonoids concentration was determined according to the methods described by Laulloo et al. (2018) and Saraiva et al. (2018), with some adaptations. For the test, a stock solution of 1,000 µg/mL was prepared for each sample by dissolving the methanolic and hydroalcoholic extracts in ultrapure water, while the hexanic extracts were dissolved in a 1:9 (v/v) hexane-methanol solution. Subsequently, samples were transferred to clean test tubes, protected from the light, and mixed with an aqueous solution of aluminum chloride (200 mg/mL) and acetic acid (P.A.), at a ratio of 1:1:0.1 (v/v/v). Finally, 2.3 mL of ethanol were added to this solution. The mixture was left to rest at room temperature for 40 minutes, and the absorbance was measured at 450 nm in a spectrophotometer. The flavonoids content in the samples was calculated according to the regression equation obtained with the calibration curve using quercetin as standard. All the determinations were carried out in triplicate, and the results were expressed in total flavonoids percentage.

2.4.2 Determination of total phenolic compounds content

Total phenolic compounds were determined according to the methods described by Laulloo et al. (2018) and Rondón et al. (2018), with some modifications. A stock solution of 1,000 µg/mL was prepared for all the samples by dissolving the methanolic and hydroalcoholic extracts in ultrapure water and the hexanic extracts in a solution of hexane + methanol (1:9, v/v). After that, the extracts were transferred to clean test tubes, protected from the light, and exposed to Folin Ciocalteau reagent and ultrapure water at a proportion of 1:1:9 (v/v/v). This solution was left to react for 5 minutes, and, subsequently, 1,000µL of sodium carbonate (7.5%) and 400 µL of ultrapure water were also added. The mixture was left to rest for 90 minutes, and the absorbance was measured at 750 nm. The total phenolics compounds content in the samples was calculated according to the regression equation obtained with a calibration curve, using gallic acid as a standard. All the tests were conducted in triplicate, and all the results were expressed as total phenolics compounds percentage.

2.5 Fourier-Transform Infrared spectroscopy

The FT-IR analysis of the liquid or semi-solid extracts was performed with an ALPHA II (Bruker) infrared spectrometer. The samples were deposited on the diamond crystal to cover its entire surface. The analysis was performed in the platinum-ATR module, with 20 scans per samples, between 4,000-500 cm^{-1}.

2.6 In vitro antiproliferative activity

The cytotoxic potential of the extracts was evaluated against three tumor cells lines using MTT assay: HCT-116 (human colorectal carcinoma), B16-F10 (murine metastatic melanoma) and HL-60 (acute myeloid leukemia). The cell lineages were previously cultivated in DMEM or RPMI-1640, supplemented with 10% of fetal bovine serum, 100 U/mL of penicillin and 100 µg/mL of streptomycin, at 37 ºC with 5% CO_2 atmosphere.

For the test, cells were seeded in a 96-well microplate at a density of 0.6 x 10^5 cells/mL (B16-F10 and HCT-116) and 3 x 10^5 cells/mL (HL-60) and incubated at 37 ºC and 5% CO_2 for 24 hours. After that, cells were treated with the extracts at a single concentration of 50 µg/mL and incubated again under the same conditions described for 72 hours. Then, the culture medium was removed, a MTT solution (0,5 mg/mL) was added to each well, and the plates were incubated at 37°C and 5% CO_2, for 3 hours. Subsequently, the formazan crystals formed were solubilized by DMSO. Tumor cell growth was quantified by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to a purple formazan product by metabolically active cells (Berridge et al., 2005). The absorbance was measured using an ELISA plate reader at 595 nm. Statistical analysis was performed using the software GraphPad Prism 6. The percentage of

cell growth inhibition of cells treated with the extracts was calculated from the cell growth values of untreated cells. Data expressed as mean ± standard deviation.

2.7 Antibacterial assay

The antibacterial activity of extracts was evaluated against *Staphylococcus aureus* (ATCC 29213) and *Escherichia coli* (ATCC 25922). The experiments were carried out according to the CLSI (2015) recommendations. First, bacterial strains were grown in Mueller Hinton agar and incubated in a bacteriological incubator at 35 ± 2 ºC for 24 hours. After that, isolated colonies were collected to produce a bacterial suspension comprising 1 - 2 x 10^8 CFU/mL (0.5 McFarland scale). This suspension was used to prepare the inoculum ($5x10^5$ CFU/mL of each strain) and perform the MIC assay.

The MIC assay was performed in a 96-well plate, using a serial microdilution method. The bacterial strains were exposed to concentrations of the extracts ranging from 5 mg/mL to 0.078 mg/mL, dissolved in sterile Mueller-Hinton broth. After the treatment, the plates were incubated at 35± 2ºC for 24 hours. The MIC was defined as the lowest concentration of the extracts able to inhibit the visible bacterial growth.

To determine the Minimum Bactericidal Concentration, 10 µL of the wells containing concentrations equal to or greater than the MIC were subcultivated in Muller-Hinton agar. MBC was defined as the lowest concentration in which no bacterial growth was observed. In both tests, the microorganisms were incubated for 24 hours, at 35±2 ºC, in aerobic conditions. All the assays were performed in triplicate.

2.8 Determination of antioxidant activity

The antioxidant activity was performed by free radical scavenging methods, using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)] (ABTS), according to the method described by Pires et al. (2017), with adaptations.

2.8.1 Antioxidant activity by the DPPH free radical method

Samples were solubilized in methanol, to prepare a stock solution of 15 mg/mL. Serial microdilutions were performed in 96-well plates, so that the concentrations tested varied between 1,000 and 15.6 µg/mL. A methanolic solution of DPPH (80 µM) was prepared and diluted to obtain an absorbance between 0.7 and 1.0 at 517 nm. Subsequently, the different concentrations of each sample were added to the wells, followed by the DPPH radical, at a 1:15 (v/v) ratio, and left to react for 30 minutes. After the reaction time, absorbance decrease was monitored at 517 nm, in an Elisa microplate reader. The test was performed in triplicate under dim light. Trolox was used as an antioxidant reference. The results were analyzed using the software GraphPad Prism 6 and expressed as IC_{50}.

2.8.2 Antioxidant activity by the ABTS free radical cation capture method

The radical solution was prepared by the reaction of ABTS (7 mM) with sodium persulfate (140 mM), for 16 hours, at room temperature. Then, the solution was filtered in with a nylon syringe filter (45 µm) and diluted in ultrapure water to obtain an absorbance between 0.8 and 1.0 at 734 nm. For the test, samples were prepared and diluted as described before, to obtain concentrations varying from 1,000 and 15.6 µg/mL. After that, the different concentrations of each sample were added to each well of a 96-well plate, followed by the radical, at a 1:15 (v/v) ratio. The test was performed in triplicate and the plate was protected from the light. After 10 minutes of reaction, the absorbance was measured in an Elisa plate reader at 734 nm. Trolox was used as an antioxidant reference. The results were analyzed in GraphPad Prism 6 and expressed as IC_{50}.

3. Results and Discussion

3.1 Phytochemical characterization

According to the results obtained by the qualitative phytochemical assay, represented in Table 1, the hexanic extracts (HXE) presented organic acids as the only secondary metabolite in the composition, independently of the collection site. These compounds were also detected in all the other extracts produced, except the HAE-PT sample. Regarding the HAE and ME extracts, Table 1 shows that their composition comprises, besides organic acids, alkaloids, phenols, tannins, and flavonoids. It is also possible to see that there is a slight variation in this composition according to the collection points, as we can see the absence of some of these compounds in determined samples.

Table 1. Qualitative analysis of the hexanic (HXE), hydroalcoholic (HAE) and methanolic (ME) extracts obtained from *M. linifera* leaves.

Samples	HXE				HAE				ME			
	PT	**LB**	**G**	**MM**	**PT**	**LB**	**G**	**MM**	**PT**	**LB**	**G**	**MM**
Saponins	-	-	-	-	-	-	-	-	-	-	-	-
Alkaloids	-	-	-	-	+	+	+	+	-	+	+	+
Organic acids	+	+	+	+	-	+	+	+	+	+	+	+
Phenols and tannins	-	-	-	-	+	-	+	-	-	+	+	+
Flavonoids	-	-	-	-	+	+	+	+	+	+	+	+
Polysaccharides	-	-	-	-	-	-	-	-	-	-	-	-

(+) = presence; (-) = absence. Source: Authors (2022).

Table 2, in turn, represents the results obtained for the quantitative analysis, showing the percentages and respective standard deviations of flavonoids and total phenolic compounds of the extracts produced.

Table 2. Percentage values of flavonoids and total phenolic compounds detected in the hexanic, hydroalcoholic, and methanolic extracts obtained from *M. linifera* leaves. Results are expressed as mean ± standard deviation.

Samples	Total Flavonoids	Total phenolic compounds
	Mean ± SD (%)	**Mean ± SD (%)**
HXE-PT	1.61 ± 0.27	3.78 ± 0.16
HXE-LB	3.17 ± 0.35	4.29 ± 0.59
HXE-G	3.93 ± 0.24	7.15 ± 0.56
HXE-MM	2.59 ± 0.22	6.63 ± 0.02
HAE-PT	2.36 ± 0.05	4.47 ± 0.28
HAE-LB	2.80 ± 0.03	4.05 ± 0.06
HAE-G	2.12 ± 0.07	7.35 ± 0.55
HAE-MM	2.57 ± 0.04	3.42 ± 0.08
ME-PT	2.54 ± 0.07	2.90 ± 0.09
ME-LB	2.73 ± 0.13	3.58 ± 0.13
ME-G	2.44 ± 0.12	8.80 ± 0.19
ME-MM	2.73 ± 0.15	4.05 ± 0.13

Source: Authors (2022).

Although the qualitative phytochemical analysis indicated organic acids as the only secondary metabolites in the HXE extracts, we observed the presence of flavonoids and phenolic acid derivatives by the quantitative analysis. These results agree with the reports by Lima et al. (2021), who also pointed the presence of these secondary metabolites in *M. linifera* extracts. In our study, the HXE-G sample presented the highest total flavonoids content and one of the highest phenols contents among the

samples produced. In this aspect, it is worth mentioning that the objective of the qualitative assays is mainly to check the presence or absence of some constituents in the natural extracts, and some methods seem to be more sensitive than others. Aziz (2015), for example, performed the qualitative phytochemical screening of the same extract by different methods, and observed that some of these methods indicated the presence of some compounds, while others showed their absence. Thus, more robust techniques, are necessary to elucidate the phytochemical composition of the samples.

However, both qualitative and quantitative methods demonstrated that the presence or absence of different secondary metabolites vary according to the locations. In this context, it is worth to consider that the plant collection sites in our study are exposed to different daily and monthly temperatures, seasonality, humidity, solar radiation, soil chemical composition, as well as the presence of predators and insects that may stimulate the production of certain active compounds (Gobbo-Neto and Lopes, 2007; Pretti et al., 2018).

Besides, it is also worth highlighting that different solvents and extraction methods may also interfere with the composition and, consequently, the biological activity of the extracts (Dirar et al., 2019). Since the polarity of the solvents plays a fundamental role in the selectivity of the extracted compounds, it is essential to choose the appropriate solvent and extraction method according to the purpose of the study (Ngo et al., 2017). Costa and Hoscheid (2018), for example, in a research conducted with the aqueous and the ethanolic extracts of *Cecropia pachystachya*, observed that the use of ethanol, a solvent with polar characteristics, like methanol, fostered the obtainment of phenolic metabolites during the extraction process. Milani et al. (2012), in turn, demonstrated that the hydroalcoholic extract of *Diospyros kaki* L. showed high levels of phenolic compounds and good antioxidant activity by the DPPH free radical method. In the present study, higher levels of phenolic compounds were obtained for ME-G and HAE-G, which were produced using two polar solvents, corroborating with the studies mentioned.

All in all, the phytochemical analysis of the extracts produced in the present research demonstrated that all the samples are composed by classes of secondary metabolites that present a range of biological applications. Organic acids, for example, show antimicrobial and antioxidant potential (Usberco and Salvador, 2006; Atkins and Jones, 2012), besides being used as additives in the food industry, acting as buffers or neutralizing agents (Fiorucci et al., 2002). These compounds are also important in the dynamics between soil and plant, since they can contribute to the soil composition, reduce the toxicity of several metal ions, provide nutrients to plants and foster the synthesis of primary metabolites, such as carbohydrates (Pinheiro et al., 2013; Schmitt et al., 2018).

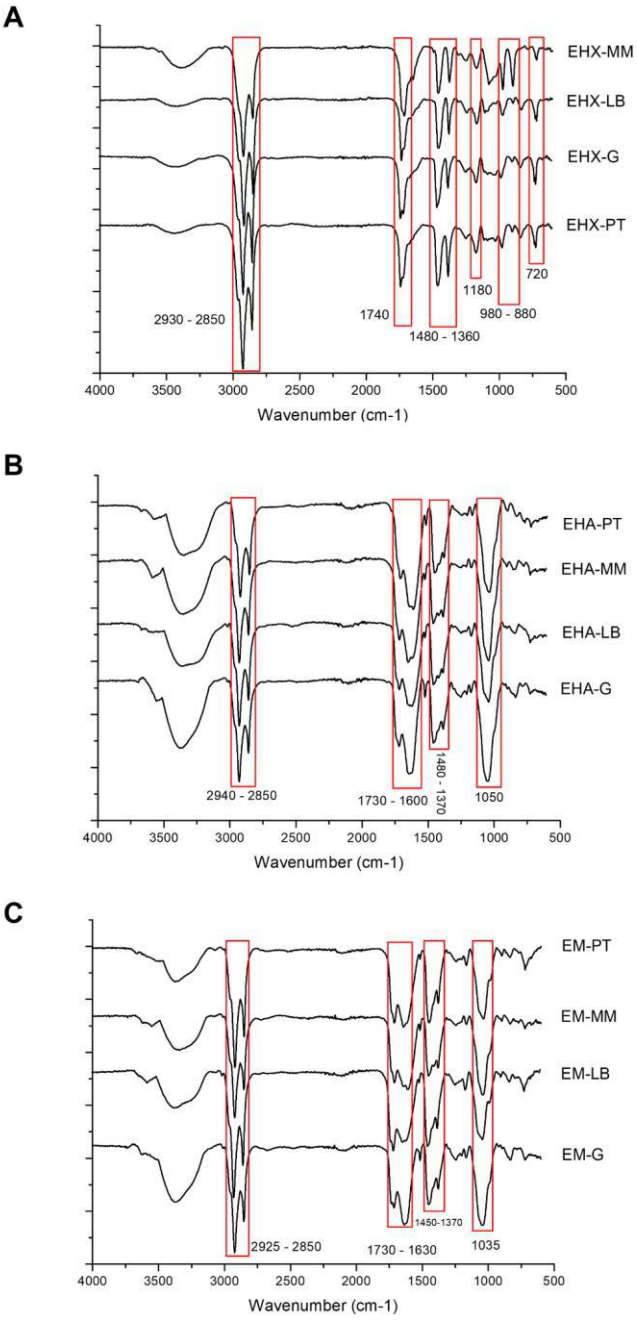
Alkaloids and phenolic compounds (phenolic acids, flavonoids, and tannins), which were also detected in the extracts produced, have been reported as anticancer, antioxidant, antimicrobial, and anti-inflammatory agents (Cupersmid et al., 2012; Brandão et al., 2017; Cole et al., 2019; Machado et al., 2020). Thus, these results may indicate that the different extracts obtained from *M. linifera* in the present research may demonstrate entrancing biological potential.

3.2 FT-IR analysis

Figure 2a demonstrates the FTIR spectra of the hexanic extracts of *M. linifera*. The spectra exhibited a characteristic absorption band at 3410 cm^{-1} associated with the stretching vibrations of alcoholic O-H or phenolic ArO-H groups. The peaks between 2930 and 2850 cm^{-1} refer to the presence of the stretching vibration of alkanes C-H of -CH3, alkanes -CH2- group, and carboxylic acids RCO-OH. The peaks in 1748 and 1717 cm^{-1} corresponds the carboxylic acid group (C=O stretch). We also observed peaks between 1458 and 1370 cm^{-1}, which may be related to the presence of C=C-C Aromatic ring stretching and O-H bending of alcoholic groups, respectively, due to the presence of Phenol or tertiary alcohol. The band at 1180 cm^{-1} represents the presence of C-O stretching associated with ether groups. The region between 980 and 880 cm^{-1} exhibited peaks related to the presence of esters (S-OR stretch), amines (N-H stretch), alkyl halide (C-Cl stretch), and alkene (= C-H bending).

The peak at 720 cm^{-1}, in turn, indicates the presence of C-Cl stretching, possibly due to the presence of aliphatic chloral compounds. The spectra of HXE-MM distinguished from the others by the presence of the bands at 967 and 890 cm^{-1}, which can be assigned to P-H phosphine bending, P-O-R esters, or N-O amine oxides. These bands could also be related to aromatic 1,3,5 trisubstituted C-H out of plane, aromatic 1,2,4,5 tetrasubstituted C-H out of plane, S-OR esters, alkenes =C-H out of plane, alkyl halides C-Cl and aromatic para disubstituted stretching. The presence of characteristic functional groups of alcohols, phenols, alkanes, esters, and alkyl halide may be associated with different secondary metabolites, which are responsible for the biological properties displayed by natural extracts (Devi and Battu 2019; Gawade, 2020).

Figure 2. FT-IR spectra of the Hexanic (a), hydroalcoholic (b), and methanolic (c) extracts of *M. linifera*.



Source: Authors (2022).

Concerning the hydroalcoholic extracts, the FTIR spectra showed no difference between the samples, as shown in figure 2b. The region between 3100 and 3500 cm^{-1} is attributed to O-H stretch vibration. The peak at 3370 cm^{-1} can be assigned to the presence alcohols and phenols (O–H stretch, H–bonded). The bands at 2940 cm^{-1} and 2850 cm^{-1} refer to the C-H stretching vibration of the -CH_3 and -CH_2-groups of alkanes and RCO-OH of carboxylic acids. The bands between 1705 and 1630 cm^{-1} may be related to the stretching vibrations of R-CO-O groups of carboxylic acids and Ar-C-C of aromatic rings. The peak at 1610 cm^{-1} denotes the presence of primary amines (N–H bend). The band at 1379.10 cm^{-1} may be due to alkanes -CH_2- and -CH_3 groups, aliphatic nitro compounds N-O, and S=O sulphate ester stretching. Finally, the bands observed in the region of 1040 cm^{-1} are possibly related to the amines with C-N stretch, or P=O group of phosphonates and phosphoramide, P-H phosphine bending, and C-O group of ethers. These groups are related to the presence of several metabolites and bioactive compounds such as flavonoids and phenolic compounds (Naumann et al., 2010; Raju et al., 2017; Gawade, 2020; Paraíso et al., 2020).

Figure 2c, in turn, shows the FT-IR spectra obtained for the methanolic extracts. It is possible to observe that the samples did not show differences in intensity. The broad bands at 2925 - 2850, 1730 - 1630, 1450 – 1370, and 1035 cm^{-1} represent the presence of functional groups, such as alcohols, phenols (OH section, linked to H), carboxylic acids (OH section), aromatics (C=C stretch (in the ring), C = C, aliphatic amines (C-N stretch) esters, and ethers (C-O stretch) (Pakkirisamy et al., 2017; Raju et al., 2017).

These results show that all the extracts analyzed demonstrated the presence of functional groups usually associated with the composition of secondary metabolites with medicinal properties, which may be responsible for the biological potential of these extracts, such as antimicrobial and anticancer activities (Raju et al., 2017).

3.3 Evaluation of the in vitro cytotoxic activity

Cancer represents one of the most important causes of death in the world. In Brazil, 600,000 new cancer cases were registered between 2018 and 2019 (INCA, 2019; INCA, 2020). The current scenario points to the necessity to search for new therapeutic alternatives with lower toxicity and, for this reason, studies that evaluate the cytotoxic potential of natural products are relevant. Plant extracts are valuable sources of compounds with potential to become new anticancer drugs, contributing considerably to the progress of cancer chemotherapy (Zhang et al., 2011; Pan et al., 2013; Vieira et al., 2017).

The cytotoxic activity of the extracts against tumor cell lines was evaluated by the MTT method, and the results obtained are expressed in Table 3. It is possible to observe that, except for HAE-MM and ME-PT, all the extracts showed cytotoxic activity against at least one of the three cancer cell lines tested, with a percentage of inhibition higher than 75%.

Table 3. Percentage of *in vitro* tumor growth inhibition after 72 hours of treatment with a single concentration of 50 µg/mL of the different extracts obtained from the leaves of *M. linifera*, determined by the MTT method.

Samples	HL-60	HCT-116	B16-F10
	Mean ± SD (%)	Mean ± SD (%)	Mean ± SD (%)
HXE-PT	57.30 ± 3.36	98.84 ± 0.14	101.25 ± 0.00
HXE-LB	34.04 ± 1.46	76.29 ± 10.58	94.40 ± 0.06
HXE-G	88.85 ± 0.84	89.83 ± 0.11	95.12 ± 0.95
HXE-MM	57.46 ± 2.35	93.24 ± 0.82	97.76 ± 0.00
HAE-PT	35.68 ± 3.01	86.89 ± 6.04	94.44 ± 0.63
HAE-LB	35.75 ± 1.54	70.14 ± 10.90	92.38 ± 0.76
HAE-G	38.07 ± 3.31	60.97 ± 8.38	78.72 ± 0.32
HAE-MM	43.44 ± 4.04	55.60 ± 4.97	71.46 ± 1.71
ME-PT	43.70 ± 5.23	47.29 ± 19.00	56.05 ± 4.50
ME-LB	31.49 ± 8.79	67.95 ± 8.45	89.87 ± 0.89
ME-G	53.39 ± 6.42	68.16 ± 8.88	91.13 ± 2.53
ME-MM	40.27 ± 2.53	63.11 ± 14.88	80.02 ± 0.63

Data expressed as mean ± standard deviation. Source: Authors (2022).

In recent years, scientific advance in chemical and pharmacological studies of medicinal plants has been considered to obtain new compounds with therapeutic properties (Casanova and Costa, 2017). Regarding the antitumor activity of natural products, Konan et al. (2012) reported the cytotoxicity of natural flavonoids against aggressive tumors-related cell lines.

In this study, the HXE-G sample was cytotoxic against the three tumor cell lines tested. The highest total flavonoids content may have contributed to the expressive results observed. Furthermore, the other samples also showed attractive cytotoxic activity, mainly against HCT-116 and B16-F10 cell lines. This potential may be associated with the flavonoids and phenolic compounds contents, previously demonstrated for the extracts obtained in this study.

Indeed, studies about the phytochemical profile of extracts obtained from *M. linifera* have described the presence of compounds that have already been reported in the literature for presenting anticancer potential, such as alkaloids (Barrales-Cureño, 2015), flavonoids (Martinez-Perez et al., 2014), and phenols (Abdal Dayen et al., 2016; Moore et al., 2016). In the present study, the qualitative phytochemical characterization indicated the presence of these compounds in the extracts produced, suggesting that they may be related to the cytotoxic activity observed.

To the best of our knowledge, this research is a pioneer in the study of the *in vitro* cytotoxic activity of the extracts obtained from *M. linifera* leaves. The results found are compatible with those found in the literature for plants with similar phytochemical composition and indicate that the samples tested contain substances with potential antitumor activity. In this aspect, *M. linifera* leaves extracts seem to be a promising resource of antitumor compounds.

Afifi and Abu-Dahab (2012) isolated the flavonoid Luteolin from *Eminium spiculatum* (Blume) Kuntze (Araceae Family) and observed antiproliferative activity against breast cancer lineages, as well as a moderate antibacterial effect against *Escherichia coli* and a resistant strain of *Staphylococcus aureus*. Thus, considering that flavonoids are phenolic compounds and were detected in the extracts by the quantitative phytochemical assay, we decided to investigate the antimicrobial potential of the samples obtained in this study.

3.4 Evaluation of the antibacterial activity

The indiscriminate use of antimicrobial drugs has favored the emergence of microbial resistance, which has become a concern among health professionals. In this aspect, the prospection of natural compounds with antibacterial potential has stood out once they can provide alternatives for the treatment of infectious diseases (Baptista, 2017; Krummenauer et al., 2019).

Among the extracts produced in the present study, the sample HXE-MM was the only one to present antibacterial

activity against the two strains tested. This sample had MIC values of 2.5 mg/mL and 1.25 mg/mL for *S. aureus* and *E. coli*, respectively, as shown in Table 4. These results demonstrate that the HXE-MM sample inhibited bacterial growth. When observing the MBC results, it is also possible to conclude that this sample displays a bacteriostatic effect against Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus*) strains. Again, the phenolic compounds and flavonoids present in this sample may have contributed to the result obtained.

Table 4. Values corresponding to the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *M. linifera* extracts against strains of *S. aureus* and *E. coli*.

Samples	*Staphylococcus aureus*		*Escherichia coli*	
	MIC (mg/mL)	**MBC (mg/mL)**	**MIC (mg/mL)**	**MBC (mg/mL)**
HXE-PT	>5	-	>5	-
HXE-LB	>5	-	>5	-
HXE-G	>5	-	>5	-
HXE-MM	2.5	-	1.25	2.5
HAE-PT	>5	-	>5	-
HAE-LB	>5	-	>5	-
HAE-G	>5	-	>5	-
HAE-MM	>5	-	>5	-
ME-PT	>5	-	>5	-
ME-LB	>5	-	>5	-
ME-G	>5	-	>5	-
ME-MM	>5	-	>5	-

Source: Authors (2022).

In the study performed by Santos et al. (2014), the methanolic extracts obtained from *M. linifera* leaves and stems showed antibacterial activity against the strain *Aeromonas hydrophila*. Amarante et al. (2010) tested different extracts of *M. linifera* against the species *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* by the agar diffusion method, but no inhibition halos were observed, indicating the absence of antibacterial activity against these strains. Miranda et al. (2015), in turn, evaluated the antibacterial potential of ethanolic and methanolic extracts obtained from the fresh and dry leaves of *M. linifera* against several bacterial strains. The authors reported that the ethanolic extract produced from the dry leaves had a moderate activity against *E. faecalis.* In the same study, the methanolic extract produced with the dry leaves was also moderately active against *S. aureus*, *S. epidermidis*, and *E. faecalis*.

Miranda et al. (2015) also demonstrated that the ethanolic and methanolic extracts obtained from the fresh leaves of *M. linifera* showed antibacterial activity against Gram-positive strains but were not active against Gram-negative bacteria. This result supports that the extraction method and solvents used are important factors to be considered when evaluating the biological potential of *M. linifera* once they can contribute to the extraction of different classes and concentrations of active compounds.

The absence of antibacterial activity in the other samples in this study may be attributed to different aspects. Once the plants grew in different localities, they were probably exposed to different environmental conditions and, thus, the proportion and composition of secondary metabolites may vary according to the sample.

3.5 Antioxidant activity

There is a growing interest in evaluating the antioxidant potential of medicinal plants, once they are of great biopharmaceutical importance and may protect essential cellular elements from oxidation during oxidative stress or disease conditions (Kumar et al., 2013a, 2013b, 2019).

The antioxidant activity of the hexanic, hydroalcoholic, and methanolic extracts from all the collection points was determined by the ABTS and DPPH free radicals scavenging methods. The results are shown in Table 5. The samples that showed better antioxidant activity were ME-G and HAE-G.

In the study performed by Santos et al. (2014), extracts obtained from the leaves and stem of *M. linifera* presented high content of phenolic compounds, as well as high antioxidant and cytotoxic activities. As shown in Table 5, the extracts obtained from the leaves of *M. linifera* collected in Galego showed the best antioxidant activities, especially ME-G and HAE-G. These extracts present high levels of phenolic compounds, which have been reported in the literature as powerful antioxidants (Soares et al., 2008).

Table 5. Antioxidant activity of the hexanic, hydroalcoholic and methanolic extracts obtained from *M. linifera* leaves. The results are expressed as IC_{50} (µg/mL) ± standard deviation.

Samples	ABTS	DPPH
	Mean ± SD (%)	Mean ± SD (%)
HXE-PT	>1000*	>1000*
HXE-LB	504.6 ± 34.75*	>1000*
HXE-G	275.5 ± 17.2*	>1000*
HXE-MM	711.3 ± 34.4*	>1000*
HAE-PT	85.27 ± 0.185	228.2 ± 19.4
HAE-LB	56.87 ± 1.65	>1000*
HAE-G	53.12 ± 1.81	110.9 ± 9
HAE-MM	104.8 ± 9.045*	274.3 ± 23.25
ME-PT	103.3 ± 20.79*	213.6 ± 1.1
ME-LB	125.1 ± 10*	587.5 ± 20.2
ME-G	48.05 ± 0.32	131.4 ± 13.8
ME-MM	113 ± 5.2*	381.6 ± 57.9
Trolox	20.7 ± 1.3	117.2 ± 27.8

*Statistical significance compared to Trolox ($p< 0.05$). Source: Authors (2022).

Table 5 also shows that, among the hexanic extracts, HXE-G showed the best antioxidant activity. This extract exhibited a high content of total flavonoids according to the phytochemical characterization performed. Flavonoids are secondary metabolites that interfere with the synthesis and spread of free radicals, acting as antioxidants. (Machado et al., 2008).

Merino et al. (2015) also observed a similar correlation between the antioxidant activity and the phytochemical composition of the extracts. The authors reported that the substances present in the crude ethanolic extract and fractions obtained from the aerial parts of *Senecio westermanii* reacted with the unstable free radical DPPH. They also observed that the crude extract showed higher antioxidant activity when compared to the fractions. Its phytochemical composition comprised alkaloids, flavonoids, iridoids, and steroids/triterpenes. This correspondence between antioxidant activity and the presence of phenolic compounds and flavonoids was also observed in our study.

According to Haro et al. (2018), methanol seems to be one of the most efficient solvents for the extraction of

antioxidant constituents. The study performed by Nawaz et al. (2020), in turn, evaluated the antioxidant potential of *Phaseolus vulgaris* extracts and observed that the antioxidant activity was more efficient for the extracts prepared with polar organic solvents than for those with non-polar organic solvents, such as hexane and chloroform. This data corroborates with the results found in the present study, once the methanolic and hydroalcoholic extracts displayed higher antioxidant activity when compared to the others.

The present study showed variations between the antioxidant activity of the extracts according to the collection site and the type of solvent used. The results obtained suggest that *M. linifera* important source of biologically active molecules, and for this reason, studies about its phytochemical profile and biological activities are of great importance.

4. Conclusion

In the present study, hexanic, methanolic and hydroalcoholic extracts of *M. linifera* leaves from different collection sites were obtained. Qualitatively, these extracts had alkaloids, flavonoids, organic acids, phenols, and tannins as major constituents, with a slight variation in the composition according to the collection sites. Quantitatively, HXE-G presented the highest total flavonoids content and one of the highest phenols contents among the samples produced. ME-G and HAE-G exhibited the highest levels of phenolic compounds. Concerning the biological activity, all the extracts showed cytotoxic activity against at least one of the three cancer cell lines tested, but only HXE-MM showed an antibacterial effect. Besides, ME-G and HAE-G extracts exhibited better antioxidant activity when compared with the other extracts.

Thus, this study demonstrated that the phytochemical composition of the extracts, as well as their biological activity, may vary according to the region where the plant develops, the solvent used to produce the extracts, and the extraction process itself, demonstrating the importance of studies about the phytochemical composition of natural products.

The existence of other bioactive compounds in the extracts that were not identified or detected due to low concentrations and methodology should be considered, therefore, more detailed phytochemical analyzes are necessary in future studies, such as the use of more robust techniques, such as Liquid Chromatography of High Efficiency and Mass Spectrometry, it is still necessary to identify with more specificity the secondary metabolites in the samples and the active principles responsible for their biological potential. In addition, to evaluate the possibility of testing new in vitro tests to evaluate other biological, phytotherapeutic and biotechnological activities.

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